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A direct observation technique for evaluating sclerotium germination by *Macrophomina phaseolina* and effects of biocontrol materials on survival of sclerotia in soil

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Abstract

Germination of sclerotia of *Macrophomina phaseolina* was quantified by direct microscopic observation following application of experimental treatments *in vitro* and incubation of sclerotia in soil. To assay germination, pieces of agar containing sclerotia were macerated in dilute, liquid cornmeal agar on glass slides; thinly spread; and incubated in a saturated atmosphere for 18–22 h. Germinated sclerotia then were identified by morphological features of germ hyphae. Frequencies of germination were similar in three dilute agar media. Germination was not affected by air-drying sclerotia for 2 weeks, but it was significantly reduced after 4 weeks and greatly reduced or eliminated after 6 or 8 weeks. Survival of sclerotia for 14 days in soil was greatest at 50, 75, and 100% moisture-holding capacity, less at 0 and 25%, and least at 125% (flooded soil). Incorporation of ground poultry litter into soil at 5% by weight reduced survival of sclerotia after 13 days, and incorporation of litter at 10% nearly eliminated it. These results indicate that the direct-observation technique may be used to evaluate animal wastes and other agricultural byproducts for biocontrol activity against sclerotia of *M. phaseolina* in soil.

Key words: biocontrol, *Macrophomina phaseolina*, sclerotium germination

Introduction

Macrophomina phaseolina (Tassi) Goid. is a major soilborne fungal pathogen that infects many agronomic, horticultural, and ornamental crops [1–3]. It is unusual in that a wide variety of both monocots and dicots are parasitized. Diseases caused by *M. phaseolina* are sometimes referred to as 'charcoal rot' on account of small, black, macroscopically visible sclerotia that form in shredded, parasitized host tissue and cause an appearance of charcoal [3]. Primary infection by *M. phaseolina* usually occurs in roots, but pathogenesis and sclerotium formation may extend above ground. Collapse and death of infected plants usually is favored by hot and dry conditions, but numerous sclerotia also may form in live host tissues without visible disease symptoms [4]. Sclerotia are the resting structures of this pathogen in soil and infested plant debris [1, 2, 4, 5].

Numerous methods have been developed to quantify sclerotia of *M. phaseolina* in soil and plant debris and to study their survival [6]. Most involve dilution plating or dusting of suspensions of soil or plant material onto selective media that may contain fungicides, antibiotics, organic compounds, salts, and dyes [1, 4, 5, 7–12]. Prior to plating, samples often are dried, ground, blended, sieved, resuspended, bleached, or centrifuged in simple to elaborate procedures. Few of these methods have been compared for efficiency and sensitivity [1, 9], but most appear to favor

detection of *M. phaseolina*. In some instances, population levels as low as ≤ one sclerotium per gram of soil have been detected [7, 9].

Although most assays for sclerotia of *M. phaseolina* in soil or plant debris are based upon soil dilution plating [4, 7, 9–12], some have evaluated sclerotium germination in soil or after removal by direct microscopic observation. Smith [13] and Collins et al. [14] incubated sclerotia in soil on membranes or agar-coated glass slides; retrieved, stained and observed sclerotia; and quantified germination that had occurred in soil. Srivastava et al. [15] placed individual sclerotia on a nylon net in soil, retrieved and washed them, and quantified germination after incubation in broth. Olaya and Abawi [16] grew *M. phaseolina* on sterile toothpicks, buried those in nylon membranes in soils, retrieved toothpicks, removed and plated sclerotia, and observed germination.

Animal wastes are major liabilities that result from year-around production of cattle, swine, and poultry in confined facilities. Most animal wastes are disposed by repeated applications to agricultural lands in close proximity to production sites, but this practice may result in chemical and biological pollution of soil, water, and air [17–19]. A more positive approach to disposing of animal wastes is to find new uses for these materials and thereby convert them from liabilities into marketable assets of production.

One possible use for animal wastes is as biocontrol materials for soilborne plant diseases. Limited evidence to date indicates that animal manures may reduce or eliminate resting structures of diverse pathogens or diseases following their incorporation into soil as amendments. Examples include *Sclerotium rolfsii* Sacc. [20], *Verticillium dahliae* Kleb. [21], *Phytophthora cinnamomi* Rands [6], *Pythium graminicola* Subr. [22], *Fusarium oxysporum* Schlecht. [23], and *Rhizoctonia solani* Kuhn [24]. We are not aware of comparable reports on effects of animal manures on *M. phaseolina* or charcoal rot diseases.

This study was undertaken to develop a simple, efficient, laboratory-based, direct observation technique to evaluate survival of sclerotia of *M. phaseolina* following their incubation in soil. Effects of assay media, air-drying of sclerotia, and soil moisture levels also were determined to establish conditions suitable for its use. This technique was intended to evaluate the potential for commercial poultry litter and other animal wastes, and eventually for other agricultural, municipal, and industrial byproducts, to provide biocontrol of sclerotia of *M. phaseolina* in soil.

Materials and methods

Sources, storage, and growth of isolates of M. phaseolina

Three isolates of *M. phaseolina* from alfalfa in Mississippi were grown in plates of Difco cornmeal agar (CMA) (Difco Laboratories, Detroit, MI) containing pieces of sterile toothpicks that were autoclaved twice in distilled water and once in 10% V-8 juice [25]. Plates were sealed in Parafilm and stored at room temperature following growth and sclerotium formation. New colonies were generated by plating pieces of infested toothpick onto CMA.

Four isolates from soybean and four from cotton were received from R.E. Baird, Department of Entomology and Plant Pathology, Mississippi State University, following storage for 1–2 years on potato dextrose agar in 10% glycerol in liquid nitrogen at −80 °C. Colonies were generated by plating isolates onto Difco potato dextrose agar (PDA).

Sclerotium formation on agar media with and without index card pieces

Sclerotium formation by *M. phaseolina* was compared on four agar media to identify conditions for producing suitable sclerotia for use in germination assays. Ten isolates, including two from alfalfa, four from cotton, and four from soybean, were each transferred to centers of four plates of PDA, CMA, Difco Czapek's agar (CZA), and water agar (20 g/l). Pieces of index card (0.25 cm^2) were autoclaved in glass plates, and four pieces were placed at equidistant points midway from the center to the edge in each plate. Plates were sealed in Parafilm and incubated for 2–3 weeks. Mean numbers of sclerotia present on the colony surface beneath two randomly selected index card pieces in each plate, and in two similar areas without index card pieces, were determined by observation at X40.

Quantification of sclerotium germination by direct microscopic observation

Sclerotia from a mixed culture of three isolates of *M. phaseolina* from alfalfa [25] were used in all experiments. The following standardized procedure was developed to assay sclerotia for germination: agar containing sclerotia was excised from beneath a piece of index card on the surface of a colony grown on thinly poured cornmeal agar (10 ml per 9.5-cm diameter petri dish). The agar with sclerotia was macerated in 0.1 ml of a liquid solution of 1/10-strength CMA (0.17 g/100 ml) on a standard glass slide (75 × 25 mm). Maceration was performed only with the cutting edge of a scalpel blade to minimize damage to sclerotia; maceration with the flat side of the blade crushed sclerotia. After 160 slashes with the scalpel blade to break up all large pieces of agar, 0.2 ml of additional dilute CMA was added and the macerate was thinly spread over 67–75% of the slide. The slide was immediately placed upon paperclip supports on double-layer filter paper saturated to excess with distilled water in a petri dish to provide a saturated atmosphere. After 18–22 h at 25–27 °C, coverslips (No. 1 thickness) were placed over the suspension and the slide was observed at X100. The percentage germination among 100 randomly selected, non-damaged, individually distinct and clearly visible sclerotia on each slide was determined.

Effects of dilute agar media, air-drying of sclerotia, soil moisture levels, and poultry litter amendments on germination of sclerotia and survival in soil

Influences of agar media on sclerotium germination were determined by macerating agar with sclerotia in dilute solutions of PDA, CMA, and CZA that were each prepared at 10% of recommended concentrations. Water agar (WA) was prepared at 0.2 g/100 ml.

Effects of air-drying sclerotia were determined by removing agar from beneath IC pieces on plates at biweekly intervals prior to assay and air-drying 0–8 weeks at room temperature.

To evaluate effects of soil moisture level, samples of a Savannah sandy loam soil collected beneath a mixed stand of bermudagrass (*Cynodon dactylon* [L.] Pers.), foxtail (*Setaria* sp.), and dallisgrass (*Paspalum dilatatum* Poir.) were sieved (8-mesh screen), air-dried to approximately 5% moisture by weight, and stored in sealed plastic bags at room temperature in darkness. Moisture contents were determined by weighing samples before and after oven-drying at 105 °C for 24 h. To determine moisture holding capacity (MHC), approximately 15 g of soil of known moisture content was placed within a cone of saturated, double-layer filter paper in a glass funnel, and distilled water was slowly added dropwise over the surface in measured amounts until soil was uniformly moistened and free water appeared at the bottom of the cone. The MHC of a mixture of 90% soil and 10% ground poultry litter was determined in the same manner. The MHC of filter paper packets containing nylon membranes and agar with sclerotia, used to incubate sclerotia in soil (below), was determined by applying drops of water to packets until runoff. Matric potentials of soil at different moisture contents were determined from published moisture-release data for a Savannah sandy loam soil in Mississippi [26].

Poultry litter, which consisted of a caked mixture of manure, feathers, and bedding (pinewood shavings and sawdust) [27], was collected from a commercial production house in Mississippi, air-dried, ground in a Wiley Mill to pass a 20-mesh screen, and stored in plastic bags in a freezer for up to 2 years. Litter was mixed with the soil to provide concentrations of 5 and 10% air-dry weight equivalent immediately prior to experimentation. Moisture-holding capacities of soil mixtures with 5 and 10% litter were determined as with unamended soil. Water was added to experimental units (below) to provide 70% moisture-holding capacity.

Incubation and retrieval of sclerotia from soil

Pieces of agar with sclerotia from beneath index card pieces on CMA were placed within folded porous nylon membranes (2 × 3 cm, 5 μm pore size) (Osmonics, Inc., Minnetonka, MN), and edges were affixed with staples. Nylon membranes were placed within folded filter paper (3 × 4 cm) and edges were affixed with staples. The filter paper provided physical protection to nylon membranes and prevented ingress of soil particles. Soil equivalent to 3.0 g oven-dry weight was placed in a small (3.5 cm diameter) plastic petri dish, a filter paper packet containing the nylon

membrane and sclerotia was placed on the soil, 3 g of additional soil was placed over the packet, and distilled water was added to bring contents of the plate up to the desired moisture level (percentage of MHC). Plates then were sealed in Parafilm and incubated on the laboratory bench at 23–25 °C.

Following incubation, filter paper packets were removed from soil in plates by gentle washing, nylon membranes were lightly blotted in tissue paper, agar containing sclerotia was lifted or scraped onto slides, and sclerotia were assayed for germination.

Experimental design and statistical analysis

All experiments were performed with plates of soil and slides of macerated sclerotia arranged in randomized complete block designs on the laboratory bench. To compare formation of sclerotia on agar with and without index card pieces, comparisons were made separately for each isolate of *M. phaseolina* on each medium. To quantify sclerotium germination, 100 randomly selected and clearly visible sclerotia were observed for germination in each experimental unit. Treatment means were compared by analysis of variance following arcsin-square root transformation of percentage data. Means that differed significantly at $P = 0.05$ were identified by Fisher's protected LSD test [28]. Results of experiments were combined if heterogeneity of variance and experiment × treatment interactions were not significant at $P = 0.05$.

Results

Production of sclerotia on agar media with and without index card pieces

Nine isolates of *M. phaseolina* from cotton, soybean, and alfalfa varied greatly in the quantity and morphology of sclerotia produced on PDA and CZA. Some isolates produced numerous, globose, individual sclerotia throughout PDA that would have been suitable for use in germination assays. However, most isolates produced sclerotia on PDA and CZA only in a thick mat of hyphae on the agar surface. These sclerotia were often complex, elongated structures with indistinctly delimited globose swellings and were not considered suitable for use to quantify germination. Sclerotium production on PDA and CZA usually was not greater beneath index card pieces than in agar alone (data not presented).

On CMA and WA, the nine isolates produced few sclerotia on agar alone, but 3–453-fold mean increases in numbers of sclerotia were observed beneath index card pieces on CMA and 1–198-fold increases were observed beneath index card pieces on WA. Increases were significant ($P = 0.05$) for eight of the nine isolates (data not presented). Sclerotia formed beneath index card pieces on CMA and WA usually were well-defined, individually delimited, globose structures present individually or in small botryose clusters. Most of these sclerotia were considered suitable for use in germination assays.

Morphology of sclerotium germination

Sclerotia that germinated in dilute CMA on slides after 18–24 h were identified by morphological features of germ hyphae (Figures 1, 2, 3). Most sclerotia produced one (Figure 1c) to four (Figures 1a, b, d, 2a, c) germ hyphae that often extended up to several hundred micrometers from sclerotia (Figure 2c). These were relatively large, broad, or stout (Figures 1a, 2a); blunt or hemispherical at tips (Figure 1a); and sparingly branched or unbranched even after extensive elongation in the dilute agar (Figure 2a–c). Germ hyphae also usually grew in distinctive, broadly curved or looping patterns (Figure 2a, c). Hyphae of other fungi present among sclerotia retrieved from soil were more narrow, acicular, much more profusely and complexly branched (Figures 3a, b), often with a spiky or kinky appearance (Figure 3c), and without characteristic growth patterns.

In quantifying sclerotium germination by *M. phaseolina*, hyphae that grew from viable cells of *M. phaseolina* itself, on and around sclerotia, posed a greater problem than did hyphae of other fungi. Growth from hyphal fragments of *M. phaseolina* occurred most commonly when sclerotia were assayed immediately after removal from plates. However, when agar pieces with sclerotia were air-dried for several days prior to assay, growth from hyphae was greatly reduced or eliminated while sclerotium germination was not affected.

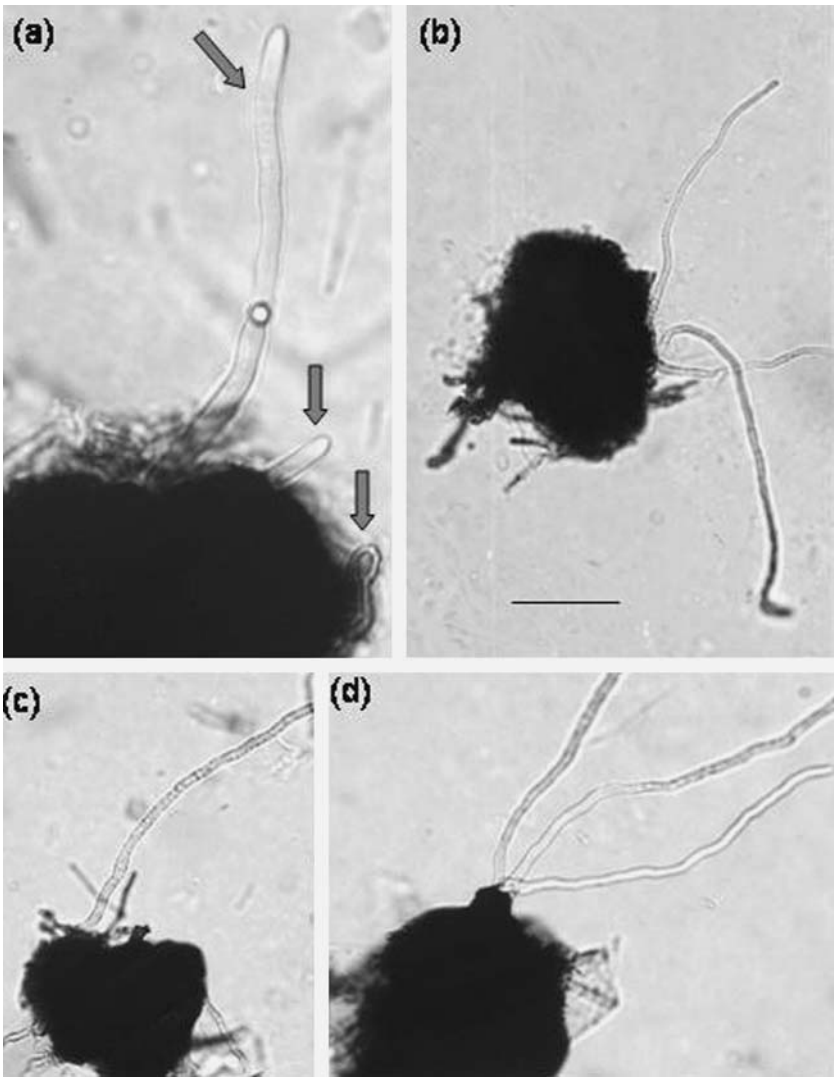

Figure 1. Germinated sclerotia of *Macrophomina phaseolina* after 18–24 h in dilute cornmeal agar. (a) Emergence of three germ hyphae from one sclerotium. Arrows indicate tips or distal portions of hyphae. (b) Three germ hyphae recently emerged and beginning extended growth. (c). Sclerotium with a single germ hypha. (d) Sclerotium with three germ hyphae emerged at the same point. Scale bar for (a), (b), (c), and (d) is 20, 50, 63, and 48 *μm,* respectively.

Effects of dilute agar media, air-drying, and moisture contents of soil on survival and germination of sclerotia

Significant differences in germination were not observed between sclerotia incubated in dilute CMA, PDA, or CZA, but germination was slightly reduced when sclerotia were incubated in dilute WA (data not presented).

In two experiments on effects of air-drying sclerotia, germination was not affected by air-drying pieces of agar containing sclerotia for 2 weeks, but less than half as many sclerotia germinated after 4 weeks, and few or none germinated after air-drying for 6 or 8 weeks (Table 1).

Frequencies of sclerotium germination following incubation in soil at six moisture levels are presented in Table 2. Maximum germination occurred with sclerotia incubated at 50, 75, and 100% MHC for 14 days with no significant differences observed between these treatments.

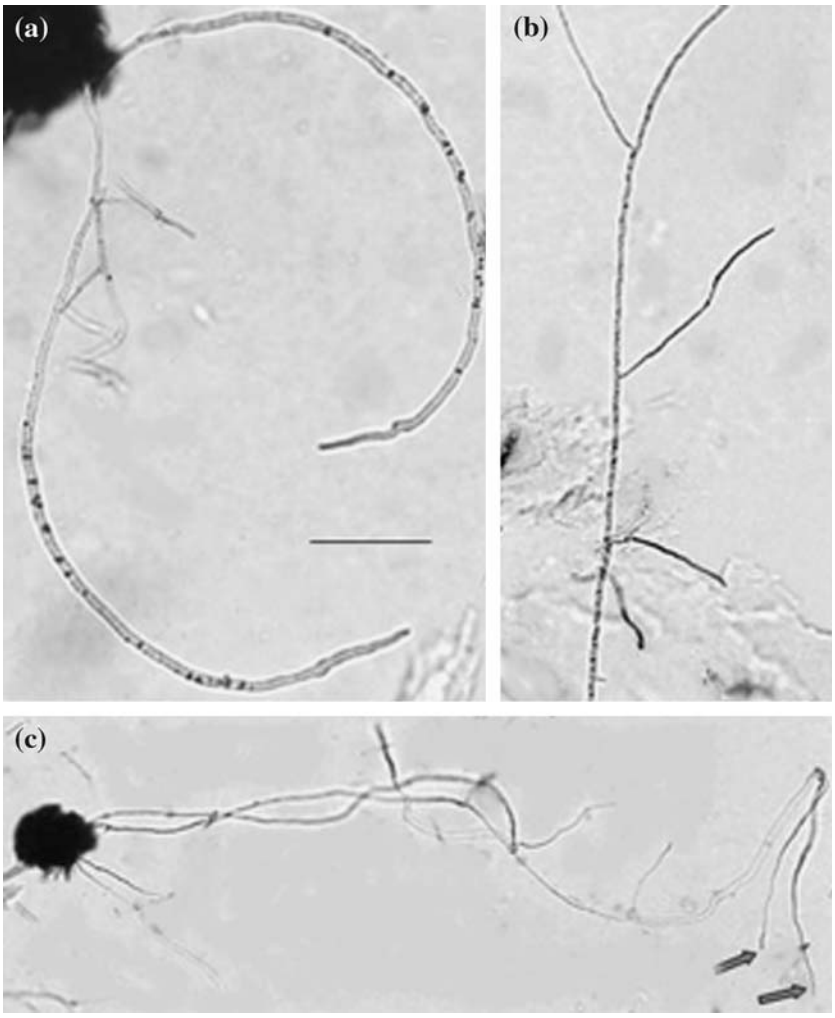

Figure 2. Appearance and characteristics of germ hyphae from sclerotia of *Macrophomina phaseolina* after incubation in dilute cornmeal agar for 24 h. (a) Two germ hyphae from one sclerotium that manifest a typical curved, broadly looping growth pattern. (b) Germ hypha with curved, broadly undulating growth habit and sparse branching. (c) Near-maximal extension of germ hypha after 24 h. Arrows indicate growing tips of a single branched hypha. Scale bar for (a), (b), and (c) is 50, 109, and 500 *μm,* respectively.

Germination was significantly reduced among sclerotia incubated at 0% MHC (air-dried soil), was further reduced at 25% MHC, and was least at 125% MHC (Table 2).

Differences between moisture-level treatments in growth of other fungi and bacteria among sclerotia also were clearly evident in both experiments. Hyphae of other fungi, and apparent chlamydospores of *Fusarium* spp., were much more profuse among sclerotia from the 25% MHC treatment than from all others. Little bacterial growth was evident from the 0 and 25% MHC treatments, but ample bacterial growth occurred at higher moisture levels.

Effects of poultry litter on survival of sclerotia in soil

Frequencies of germination of sclerotia following incubation for 13 days in soil with and without ground poultry litter amendments are presented in Table 3. Incorporation of litter at 5% by weight resulted in approximately a 75% decrease in survival of sclerotia in comparison to unamended soil. Increasing the litter content to 10% resulted in a further 75% decrease that nearly eliminated survival of sclerotia.

Sclerotia retrieved from soils amended with poultry litter were physically intact, of normal color, and did not differ consistently in appearance

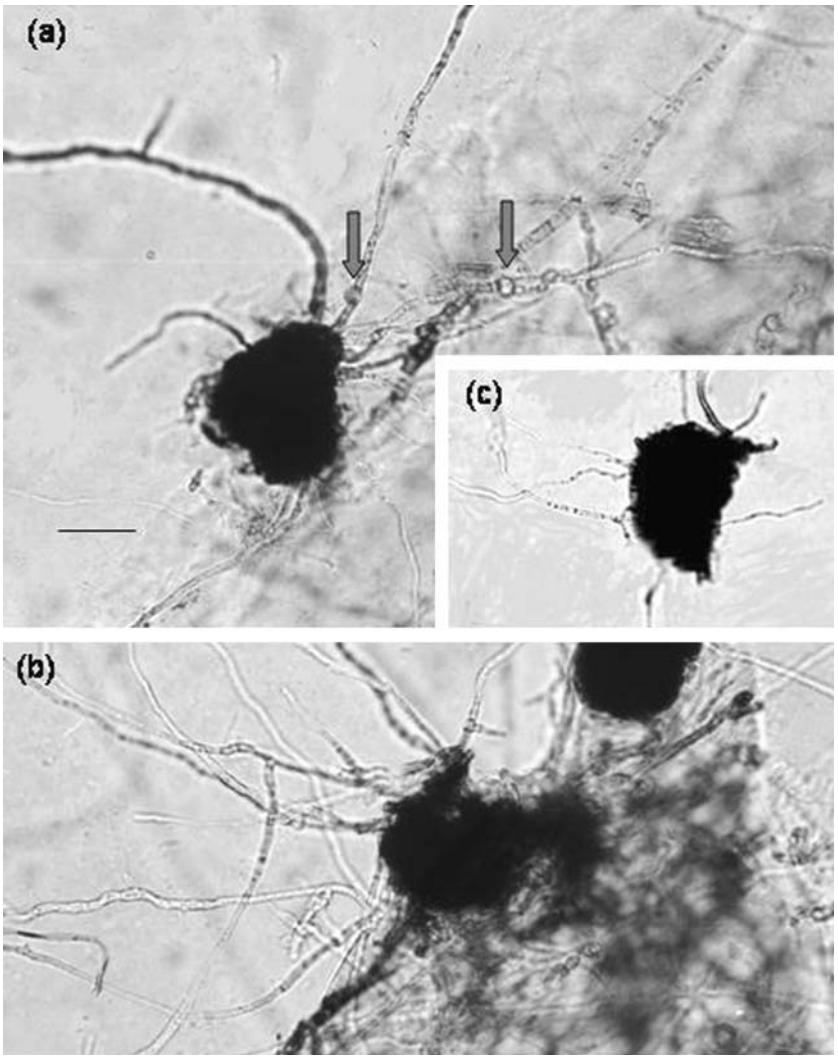

Figure 3. Growth of saprophytic or mycoparasitic fungi from sclerotia of *Macrophomina phaseolina* after incubation in soil amended with ground poultry litter. (a) Growth of numerous hyphae from a sclerotium. Arrows indicate chlamydospores similar to those of *Fusarium* spp. formed within hyphae. (b) Growth of numerous hyphae with frequent branching in vicinity of sclerotium. (c) Narrow, spiky-appearing hyphae grown from a sclerotium. Scale bar for (a), (b), and (c) is 50, 37, and 52 *μm,* respectively.

from sclerotia retrieved from unamended soil. However, during germination assays, hyphae of *Fusarium* and other fungi developed much more profusely from and around sclerotia retrieved from litter-amended soil than from unamended soil.

Discussion

Results of this study demonstrate that the direct observation technique is a useful method for evaluating germination of sclerotia of *M. phaseolina* after exposure to physical treatments *in vitro* or following incubation in soil. This technique is advantageous because large numbers of sclerotia are evaluated individually, final results are obtained within a day, and sclerotium germination is verified by direct microscopic observation (Figure 1) rather than indirectly inferred from numbers of colonies that develop on soil dilution plates [1, 4, 5, 7–12]. Direct observations of sclerotium germination also may provide insights into causes for failure of sclerotia to survive in soil: observations of other microorganisms suggest that

Table 1. Germination of sclerotia of *Macrophomina phaseolina* following air-drying for 0–8 weeks[a]

Weeks air-drying following removal of agar from colonies	Mean germination (%)[b]
0	68 a
2	67 a
4	28 b
6	6 c
8	0 d

[a]Pieces of agar containing sclerotia were macerated in dilute cornmeal agar on slides, incubated in a saturated atmosphere, and observed for germination after 18–22 h.
[b]Values are mean numbers of sclerotia germinated among 100 observed in each of 4 replicate plates in each of two combined experiments. Means not followed by the same letter differ significantly at $P = 0.05$ according to Fisher's protected LSD test.

mycoparasitism, possibly by *Fusarium* spp., may reduce survival of sclerotia in soil as indicated previously (Figure 3) [14, 15].

The most valuable feature of this technique is that sclerotia in agar are macerated, spread, and incubated in a thin layer of liquid agar on glass slides following retrieval from soil. Most sclerotia that germinate in this narrow plane are clearly visible (Figure 1), and germination is verified by morphological features of hyphae that emerge from sclerotia (Figure 2). Accurately identifying germ hyphae of *M. phaseolina* is crucial because hyphae of numerous other fungi also may grow from sclerotia following incubation in soil (Figure 3) [15]; these can easily be mistaken for *M. phaseolina* if morphological features are not observed clearly and used as criteria for identification. A second beneficial feature of this technique is that most sclerotia formed in CMA beneath index card pieces are well-defined, distinct, spherical or globose structures that are more suitable for quantifying germination than the larger, broadly fused, and poorly delimited sclerotia often formed on PDA.

There are strong similarities between the present technique and that of Collins et al. [14], especially in incubation of sclerotia within agar in soil, but the Collins technique was used to evaluate germination of sclerotia *in situ* in soil rather than after retrieval. The direct-observation techniques of Smith [13] also were used to evaluate germination within soil in response to nutrients. The technique of Srivastava et al. [15] involved incubating and retrieving individual sclerotia from soil in order to isolate mycoparasites. Sclerotium germination was evaluated with this technique in one experiment [15], but it does not appear efficient for quantifying germination of large numbers of sclerotia following incubation in soil. Data obtained by Olaya and Abawi [16] in their direct observation technique was based upon only small numbers of observed sclerotia, and details of how these were removed from toothpicks, plated, and observed to verify germination by *M. phaseolina* were not described.

Nylon membranes with pore sizes of 5 μm, rather than nylon mesh fabric, were used to incubate sclerotia in soil in order to reduce movement of soil particles and organic matter into preparations of sclerotia. It appeared likely that the presence of numerous extraneous particles from soil among sclerotia, as would likely occur with mesh fabric, might interfere with observations of ger-

Table 2. Germination of sclerotia of *Macrophomina phaseolina* following incubation in soil at six moisture levels[a]

Soil moisture[b]			Mean germination (%)[c]
Moisture content (%)	Moisture-holding capacity (%)	Matric potential (MPa)	
<1	0	<−1.5	46 b
6	25	<−1.5	35 c
13	50	−0.8	58 a
19	75	−0.01	63 a
25	100	0	58 a
31	125	0	17 d

[a]Pieces of agar containing sclerotia were macerated in dilute cornmeal agar on slides, incubated in a saturated atmosphere, and observed for germination after 18–22 h.
[b]Moisture-holding capacity was determined by adding water to soil in a funnel until release was observed. Matric potentials were determined from published moisture-release data for a Savannah sandy loam soil in Mississippi [26].
[c]Values are mean numbers of sclerotia germinated among 100 in each of four replicate plates in two combined experiments. Means not followed by the same letter differ significantly at $P = 0.05$ according to Fisher's protected LSD test.

mination. Even with the porous membranes used, some particles of soil and organic debris did ingress among sclerotia, but these were usually not sufficient to impede observations of sclerotium germination, growth of germ hyphae, and growth of other fungi from sclerotia (Figures 1–3). It also appears that the nylon membranes used in this study did not limit contact of soil microorganisms and fauna with sclerotia because in all preparations incubated in soil at high moisture levels (Tables 2, 3), a diverse array of fungi, bacteria, actinomycetes, nematodes, and other soil invertebrates were observed among agar pieces containing sclerotia that were retrieved from soil. Organisms larger than 5 μm diameter apparently ingressed to sclerotia through larger spaces present around loosely stapled edges of membranes. Therefore, these observations indicate that sclerotia incubated in soil by this technique appear to have been exposed to the full array of microorganism present in soil. The same observations also indicate that use of soil stored for several months at 5% moisture content did not limit the microbial populations to which sclerotia were exposed. The incubation of sclerotia in moist soil for nearly 2 weeks appears to have provided ample time for the germination and growth of diverse soil microorganisms and fauna from dormant propagules and initiation of potential interactions with the sclerotia of *M. phaseolina*.

Results of experiments on air-drying sclerotia within pieces of agar indicate that sclerotia of *M. phaseolina* survive *in vitro* for only short periods of time in an air-dried condition (Table 1). These results are very similar to those of Ayanru and Green [29], who observed drastic declines in survival of air-dried sclerotia after 30 days. Short et al. [11] and Kendig et al. [4] also described significant decreases in numbers of sclerotia in soil and plant residues in the field within periods of one to several months. However, other authors have indicated that sclerotia may survive for many weeks or even years in air-dried substrates or dry soil [1, 5, 16]. Possibly if thousands of sclerotia are present in infested substrates such as toothpicks, survival at only very low levels still may be sufficient to generate colonies when substrates are plated on agar.

Soil moisture content is considered to be a major factor that affects survival of sclerotia of *M. phaseolina* in soil. In most previous studies, survival was least in saturated soils or at the highest moisture levels [8, 16, 30]. Similarly, the least survival observed in this study was in flooded soil (125% MHC) (Table 2). However, results of this study do not clearly indicate that dry soils favor survival of sclerotia, as has been suggested by some authors [8, 16], because optimal survival occurred in relatively moist soil at 50–100% MHC (0 to −0.8 MPa matric potential) (Table 2). Papavizas also observed greater survival of sclerotia in soils at 50 and 70% MHC than at 2–30% [10], and Shokes et al. observed similar survival over a broad range of moisture levels including moist soil near field capacity [30].

Results and observations from experiments on soil moisture levels in this study suggest that complex interactions between microorganisms and soil moisture may influence survival of sclerotia. Growth of other fungi associated with sclerotia, and especially *Fusarium* spp., was much more profuse on slides from the 25% MHC treatment than from all others, and survival of sclerotia in soil at 25% MHC also was significantly less than at 0% MHC or higher moisture levels. It appears likely that at 0% MHC, moisture was insufficient to enable growth of either bacteria or fungi in soil, so microbial influences on survival of sclerotia were likely non-existent. At 25% MHC, apparently sufficient moisture was present to stimulate germination of fungal spores and growth of hyphae, but films of moisture needed to enable growth of bacteria were not present. Therefore, at 25% MHC, the profuse growth of hyphae and spores of other fungi apparently resulted from an absence of bacterial antibiosis. This uninhibited growth of other fungi may have caused greater mycoparasitism of sclerotia of *M. phaseolina* [15] and reduced their survival in comparison to most other treatments (Table 2).

Although observations of this study suggest that mycoparasitism may have accounted for the reduced survival of sclerotia in soil amended with poultry litter, it is also possible that sclerotia were stimulated to germinate in soil by nutrients released from the litter. Presumably sclerotia that germinated in soil would no longer be capable of additional germination following their retrieval from soil. Evaluation of germination in soil as a possible cause for reduced germination of sclerotia retrieved from soil would require use of an *in situ* observation technique such as those of Collins [14]

Table 3. Germination of sclerotia of *Macrophomina phaseolina* following incubation in soil with and without ground poultry litter incorporated at two rates[a]

Treatment[b]	Mean germination (%)[c]
0 litter	36 a
5% litter	9 b
10% litter	2 c

[a]Pieces of agar containing sclerotia were macerated in dilute cornmeal agar on slides, incubated in a saturated atmosphere, and observed for germination after 18–22 h.
[b]Ground poultry litter was mixed into soil at 0, 5 and 10% by weight and water was added to provide 70% moisture-holding capacity.
[c]Values are mean numbers of sclerotia germinated among 100 in each of five replicate plates in two combined experiments. Means not followed by the same letter differ significantly at $P = 0.05$ according to Fisher's protected LSD test.

and Smith [13]. Possibly such a technique could be used in conjunction with the present technique to more fully evaluate germination of sclerotia of *M. phaseolina* both in soil and following their retrieval from it.

The principal objective of this study, to develop a technique for evaluating animal wastes and other byproducts for biocontrol of sclerotia of *M. phaseolina* in soil, was fulfilled for poultry litter because adding this byproduct to soil greatly reduced or nearly eliminated survival of sclerotia after only 13 days. Therefore, these results demonstrate that poultry litter is efficaceous as a biocontrol material against sclerotia of *M. phaseolina* in soil, and they suggest that other byproducts also may be evaluated for biocontrol potential by this direct-observation technique.

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References

1. Mihail JD. Methods for research on soilborne phytopathogenic fungi. In: Singleton LL, Mihail JD, Rush CM, eds. Macrophomina. St. Paul, MN: American Phytopathological Society, 1992: 34–136.
2. Wyllie TD. Charcoal rot. In: Sinclair JB, Backman PA, eds. Compendium of Soybean Diseases, 3rd edn. St Paul, MN: American Phytopathological Society, 1989: 30–33.
3. Young PA. Charcoal rot of plants in east Texas. Bull. 712, Texas Agr. Exp. Sta., 1949: 33 pp.
4. Kendig SR, Rupe JC, Scott HD. Effect of irrigation and soil water stress on densities of *Macrophomina phaseolina* in soil and roots of two soybean cultivars. Plant Dis. 2000; 84: 895–900.
5. Papavizas GC. Some factors affecting survival of sclerotia of *Macrophomina phaseolina* in soil. Soil Biol Biochem 1977; 9: 337–341.
6. Aryantha IP, Cross R, Guest DI. Suppression of *Phytophthora cinnamomi* in potting mixes amended with uncomposted and composted animal manures. Phytopathology 2000; 90: 775–782.
7. Campbell CL, Nelson LA. Evaluation of an assay for quantifying populations of sclerotia of *Macrophomina phaseolina* from soil. Plant Dis 1986; 70: 645–647.
8. Dhingra OD, Sinclair JB. Survival of *Macrophomina phaseolina* sclerotia in soil: Effects of soil moisture, carbon:nitrogen ratios, carbon sources, and nitrogen concentrations. Phytopathology 1975; 65: 236–240.
9. Mihail JD, Alcorn SM. Quantitative recovery of *Macrophomina phaseolina* sclerotia from soil. Plant Dis 1982; 66: 662–663.
10. Papavizas GC, Klag NG. Isolation and quantitative determination of *Macrophomina phaseolina* from soil. Phytopathology 1975; 65: 182–187.
11. Short GE, Wyllie TD, Bristow PR. Survival of sclerotia of *Macrophomina phaseolina* in soil and in residue of soybean. Phytopathology 1980; 70: 13–17.
12. Singh SK, Nene YL, Reddy MV. Influence of cropping systems on *Macrophomina phaseolina* populations in soil. Plant Dis 1990; 74: 812–814.
13. Smith WH. Germination of *Macrophomina phaseolina* sclerotia as affected by *Pinus lambertiana* root exudate. Can J Microbiol 1969; 15: 1387–1391.
14. Collins DJ, Wyllie TD, Anderson SH. Biological activity of *Macrophomina phaseolina* in soil. Soil Biol Biochem 1981; 23: 495–496.
15. Srivastava AK, Arora DK, Gupta S, Pandey RR, Lee MW. Diversity of potential microbial parasites colonizing sclerotia of *Macrophomina phaseolina* in soil. Biol Fertil Soils 1996; 22: 136–140.
16. Olaya G, Abawi GS. Effect of water potential on mycelial growth and on production and germination of sclerotia of *Macrophomina phaseolina*. Plant Dis 1996; 80: 1347–1350.
17. Arogo J, Westerman PW, Heber AJ, Robarge WP, Classen JJ. Ammonia produced by animal operations. In: Havenstein GB, ed. Proc. Internat. Symp. Addressing Animal Production and Environmental Issues. Oct. 3–5, 2001, Research Triangle Park, NC. Dept. Communication Services, North Carolina State University, Raleigh, NC, 2002: 278–293.
18. Pierzynski GM, Sims JT, Vance GH. Soils and Environmental Quality. Boca Raton, FL: CRC Press, 2000: 459 pp.
19. Sharpley A, Meisinger JJ, Breeuwsma A, Sims JT, Daniel TC, Schepers JS. Impacts of animal manure management on ground and surface water quality. In: Hatfield JL,

Stewart BA, eds. Animal Waste Utilization: Effective use of Manure as a Soil Resource, Ann Arbor PressChelsea, MI, 1998: 173–242.
20. Bullock LR, Ristaino JB. Effect of synthetic and organic soil fertility amendments on southern blight, soil microbial communities, and yield of processing tomatoes. Phytopathology 2002; 92: 181–189.
21. Tenuta M, Conn KL, Lazarovits G. Volatile fatty acids in liquid swine manure can kill microsclerotia of *Verticillium dahliae*. Phytopathology 2002; 92: 548–552.
22. Craft CM, Nelson EB. Microbial properties of composts that suppress damping-off and root rot of creeping bentgrass caused by *Pythium graminicola*. Appl. Environ. Microbiol. 1996; 62: 1550–1557.
23. Wicks TJ, Volle D, Baker BT. The effect of soil fumigation and fowl manure on populations of *Fusarium oxysporum* f. sp. *cucumerum* in glasshouse soil and the incidence of cucumber wilt. Agric. Rec. South Australia Dept. Agric. Adelaide, The Department, Vol. 5, 1978: 4–8.
24. Voland RP, Epstein AH. Development of suppressiveness to diseases caused by *Rhizoctonia solani* in soils amended with composted and noncomposted manure. Plant Dis. 1994; 78: 461–466.
25. Pratt RG, McLaughlin MR, Pederson GA, Rowe DE. Pathogenicity of *Macrophomina phaseolina* to mature plant tissues of alfalfa and white clover. Plant Dis 1998; 82: 1033–1038.
26. Pettry DE, Switzer RE, Hinton RB. Temporal water table levels and characteristics of representative Mississippi soils. Bull. 1027, Mississippi Agr. & For. Exp. Sta., 1995: 88 pp.
27. Miles DM, Sistani KR. Broiler phosphorus intake versus broiler phosphorus output in the United States: Nutrition or soil science?. World's Poultry Science Journal 2002; 58: 493–500.
28. Steele RGD, Torrie JH. Principles and Procedures of Statistics. NY: McGraw-Hill, Inc, 1960: 481 pp.
29. Ayanru DKG, Green RJ, Jr. Alteration of germination patterns of sclerotia of *Macrophomina phaseolina* on soil surfaces. Phytopathology 1974; 64: 595–601.
30. Shokes FM, Lyda SD, Jordan WR. Effect of water potential on the growth and survival of *Macrophomina phaseolina*. Phytopathology 1977; 67: 239–241.

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